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Matrix Pathobiology

Matrix Contraction by Dermal Fibroblasts Requires Transforming Growth Factor-β/Activin-Linked Kinase 5, Heparan Sulfate-Containing Proteoglycans, and MEK/ERK

Insights into Pathological Scarring in Chronic Fibrotic Disease

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Scarring is characterized by excessive synthesis and contraction of extracellular matrix. Here, we show that fibroblasts from scarred (lesional) areas of patients with the chronic fibrotic disorder diffuse scleroderma [diffuse systemic sclerosis (dSSc)] show an enhanced ability to adhere to and contract extracellular matrix, relative to fibroblasts from unscarred (nonlesional) areas of dSSc patients and dermal fibroblasts from normal, healthy individuals. The contractile abilities of normal and dSSc dermal fibroblasts were suppressed by blocking heparin sulfate-containing proteoglycan biosynthesis or antagonizing transforming growth factor-β receptor type I [activin-linked kinase (ALK5)] or ras/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK). Compared with both normal and nonlesional fibroblasts, lesional dSSc fibroblasts overexpressed the heparin sulfate-containing proteoglycan syndecan 4. We also found that the procontractile signals from transforming growth factor (TGF)-β were integrated through syndecan 4 and MEK/ERK because the ability of TGFβ to induce contraction of dermal fibroblasts was prevented by MEK antagonism. TGFβ could not induce a contractile phenotype or phosphorylate ERK in *syndecan 4*$^{-/-}$ dermal fibroblasts. These results suggest that integrating TGFβ and ERK signals via syndecan 4 is essential for the contractile ability of dermal fibroblasts. We conclude that antagonizing MEK/ERK, TGFβ1/ALK5, or syndecan 4 may alleviate scarring in chronic fibrotic disease. *(Am J Pathol 2005, 167:1699–1711)*

The wound healing response to tissue injury requires the *de novo* synthesis of connective tissue. Normally, the wound healing process is appropriately terminated, and proper organ function is restored. However, it is believed that if the wound healing process continues unabated,

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fibrosis, a condition characterized by the excessive deposition and contraction of extracellular matrix (ECM), results.[1] In its less severe forms, fibrosis might consist of merely a disfiguring scar, or in a localized hyperproliferation of fibroblasts, such as a keloid. However, in its most severe forms, excessive scarring can result in fibrotic disease, which is characterized by tissue destruction, organ dysfunction, or death due to systemic organ failure. Currently, there is no effective therapy for fibrotic disease, in part because the underlying cause of these disorders remains elusive.

Fibroblasts are generally considered to be major effecter cells in fibrotic disease by contributing to the increased synthesis and contraction of extracellular matrix (ECM) characteristic of these disorders.[2,3] In the wound healing response, fibroblasts proliferate, migrate into the wound and produce increased amounts of ECM. This transformation of fibroblasts to proliferating, excessively matrix-producing cells has been termed fibroblast activation. Activated fibroblasts in the wound are often termed "myofibroblasts,"[4] because they express the procontractile protein α-smooth muscle actin (α-SMA). In fibrotic lesions, α-smooth muscle actin (α-SMA)-positive matrix-producing myofibroblasts persist.[4] Based on data observed in normal fibroblasts induced to express α-SMA in cell culture, these α-SMA-expressing fibroblasts present in fibrotic lesions would be expected to contribute to the excessive contraction and synthesis of extracellular matrix characteristic of the enhanced tensile strength of scar tissue.[5] However, the precise extent to which the lesional fibroblast is autonomously activated in chronic fibrotic disease and the phenotypic alterations in the fibroblast correlating with the progression of fibrosis have yet to be fully elucidated. Furthermore, although dysregulated transforming growth factor (TGF)-β receptor signaling has been hypothesized to play a role in chronic fibrotic disease such as scleroderma,[6–8] the precise contribution of TGFβ receptors to the pathology of fibrotic disorders remains to be elucidated.

Scleroderma [systemic sclerosis (SSc)] is a chronic disease of unknown etiology characterized by microvascular injury, autoimmune inflammatory responses, and severe and often progressive fibrosis.[2,3,9,10] Because SSc shares similar features to other fibrotic diseases, elucidating the molecular basis of SSc is likely to be beneficial in understanding the nature of fibrotic disease in general. Clinically, SSc is heterogeneous ranging form mild, limited skin sclerosis with minimal organ involvement (limited SSc), to diffuse skin involvement and severe fibrosis of multiple internal organs [diffuse SSc (dSSc)].[2,3,9,10] Mortality of dSSc patients is high and is directly related to the extent of scarring.[2,3,9,10] Clinically, dSSc skin has been characterized by "lesional" and "nonlesional" (ie, clinically affected and nonaffected) areas based on the physical appearance of appreciable scar tissue.[9] SSc dermal fibroblasts can be readily isolated and cultured and retain their ability to overexpress type I collagen and connective tissue growth factor.[11–14] Thus examination of the phenotypic—and molecular—difference among normal fibroblasts from healthy individuals and fibroblasts from nonlesional and lesional areas of dSSc patients should yield valuable insights into the molecular nature of scar tissue formation and progression in chronic fibrotic disease in general.

Consequently, to begin to understand the molecular nature of scar formation in progressive fibrotic disease, we compare the phenotypes and gene expression profiles of normal, nonlesional, and lesional dSSc dermal fibroblasts. Our results yield new insights into the molecular basis of ECM contraction by fibroblasts and suggest new methods of combating chronic, pathological fibrosis.

Materials and Methods

Cell Culture

Briefly, cell culture was performed as previously described.[13] Dermal fibroblasts from lesional and nonlesional (clinically affected and nonaffected) areas of patients with diffuse SSc (between 12 and 18 months duration) and normal individuals were taken from biopsies of age, sex, and anatomically site-matched volunteers, after informed consent and ethical approval was obtained. All patients fulfilled the criteria of the American College of Rheumatology for the diagnosis of diffuse SSc. Patients were female. None was receiving immunosuppressive medication or corticosteroids at the time of biopsy. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 mg/ml streptomycin and cultured in a humidified atmosphere of 5% CO_2 in air. Fibroblasts were subcultured 1:4 at confluence. *Syndecan* $4^{+/+}$ and *syndecan* $4^{-/-}$ dermal fibroblasts were isolated and cultured as previously described.[15] When appropriate, the MEK inhibitors U0126 (10 μmol/L; Promega, Southampton, United Kingdom) and PD98059 (30 μmol/L; Calbiochem, La Jolla, CA) or the ALK5 inhibitor SB431542 (10 μmol/L; Tocris, Bristol, United Kingdom) was added for the durations indicated.

Expression Profiling

Expression profiling was conducted as previously described.[16–18] Dermal fibroblasts from four normal individuals and dermal fibroblasts from areas of persistent fibrotic areas of lesions of three dSSc patients were cultured until confluence and serum starved for 18 hours. Fibroblasts were of passage 4. Media were changed and cultured for an additional 8 hours. At the end of the treatment period, total RNA was harvested (Trizol; Life Technologies) and quantified, and integrity was verified by denaturing gel electrophoresis. Equal amounts of identically treated RNA were pooled and reverse transcribed (Life Technologies) into cDNA that was then *in vitro* transcribed into biotinylated cRNA. The target cRNA was then fragmented and hybridized to the Affymetrix human U133A array (Affymetrix, Santa Clara, CA), following Affymetrix protocol. Hybridization of cRNA to Affymetrix human U133A chips, signal amplification, and data collection were performed using

Table 1. Proadhesive and Procontractile Genes Up-Regulated in Lesional Scleroderma Fibroblasts (>Threefold) in Normal Fibroblasts

Affymetrix ID	Matrix, cytoskeleton, and adhesion-associated genes	Accession no.	Fold increase
202620_s_at	*Procollagen-lysine 2 (PLOD2)*	NM_000935.1	6.2
209663_s_at	*Integrin α-7*	AF072132.1	5.5
204627_s_at	*Integrin, β3*	M35999	7.4
202351_at	*Integrin, αV*	AI380298	4.6
201389_at	*Integrin, α5*	NM_003461.1	4.6
212158_at	*Syndecan 2*	AL577322	4.8
202071_at	*Syndecan 4*	NM_002999.1	3
200859_x_at	*Filamin A, α (actin-binding protein-280)*	NM_014000.1	4.4
200974_at	*Actin, α2, smooth muscle, aorta*	NM_001613.1	7.1
205132_at	*Actin, α, cardiac muscle*	NM_005159.2	6.1
211126_s_at	*Smooth muscle LIM protein*	U46006.1	5.9
211823_s_at	*Paxillin β*	D86862.1	4.7
200931_s_at	*Vinculin*	NM_014000.1	4.4
200808_s_at	*Zyxin*	NM_006932.1	4.6
207390_s_at	*Smoothelin*	AL046979	4.5
221748_s_at	*Tensin*	AL046979	4.6
210764_s_at	*CYR61*	AF003114.1	5.6
209101_at	*Connective tissue growth factor*	M92934.1	6.4

an Affymetrix fluidics station and chip reader. Chip files were scaled to an average intensity of 100 per gene and analyzed using the Affymetrix version 5.0 (MAS5) comparison analysis software. Experiments were performed twice, and fold changes presented in Table 1 are an average of these independent studies. Criteria indicated by Affymetrix were used to determine robust changes in gene expression. Briefly, transcripts were defined as up-regulated in dSSc only when identified as "Present" by the Affymetrix detection algorithm and as significantly increased as determined by the Affymetrix change algorithm, with a change in *P* value of <0.01. Approximately 300 transcripts were overexpressed, whereas approximately 150 transcripts were decreased, in SSc fibroblasts. The fold change between treated and untreated samples had to be at least threefold to identify a transcript as being altered. These criteria had to be met in both sets of experiments.

Western Blot and Immunofluorescence Analysis

Cells were cultured until confluence in DMEM 10% fetal bovine serum (FBS). Cell layers were harvested using 2% sodium dodecyl sulfate (SDS). Proteins were quantified (Bradford; Bio-Rad, Hercules, CA), and equal amounts of protein (25 μg) were subjected to SDS-polyacrylamide gel electrophoresis using 4 to 12% polyacrylamide gels (Invitrogen, Carlsbad, CA). Gels were blotted onto nitrocellulose, and proteins were detected using anti-CCN2 (Santa Cruz, Santa Cruz, CA), anti-moesin, anti-paxillin, anti-vinculin, anti-ezrin (Cell Signaling, Beverly, MA), anti α-SMA (Sigma, St. Louis, MO), anti-syndecan 4, anti-syndecan 2, anti-α4 and anti-β1 integrin (Zymed, South San Francisco, CA), and appropriate horseradish peroxidase-conjugated secondary antibodies (Zymed) and an ECL kit (Amersham, Little Chalfont, United Kingdom). To detect type I collagen, equal amounts of media were precipitated with 30% ammonium sulfate, resuspended in 2% SDS, and subjected to SDS-polyacrylamide gel electrophoresis. Gels were blotted onto nitrocellulose, and type I collagen was detected with an anti-type I collagen antibody (Biodesign, Saco, ME), as described above. For immunofluorescent detection, cells were fixed in 3% paraformaldehyde (15 minutes), and localization of proteins was detected as previously described.[19] Densitometry was performed using AlphaEase (Alpha Innotech, San Leandro, CA) as previously described.[19] Expression values were calculated relative to baseline, and average ± SD was obtained. Student's paired *t*-test was performed on the protein expression obtained in dSSc fibroblasts, relative to protein expression in normal fibroblasts ($P < 0.05$).

Adhesion Assay

Fibroblasts were isolated and cultured as described above. Fibroblasts isolated from three normal individuals and lesional areas of three individuals with dSSc were assayed in triplicate. Fibroblasts were used at passage 3. Adhesion assays were performed[19] by initially coating wells of 96-well plates overnight at 4°C, with 6 mg/ml fibronectin (Sigma) in 0.5% bovine serum albumin (BSA) and 1× phosphate-buffered saline (PBS). Wells were blocked for 1 hour in 10% BSA in PBS, room temperature. Fibroblasts were harvested with 2 mmol/L ethylenediamine tetraacetic acid in PBS (20 minutes at room temperature), washed twice with DMEM serum-free medium containing 1% BSA (Sigma), and resuspended in the same medium at 2.5×10^5 cells/ml. To detect cell adhesion, an acid phosphatase assay was used, and adherent cells were quantified by incubation with 100 μl of substrate solution (0.1 mol/L sodium acetate, pH 5.5, 10 mmol/L −*p*-nitophenylphosphate, and 0.1% Triton X-100) for 2 hours at 37°C. The reaction was stopped by the addition of 15 μl of 1 N NaOH/well, and A_{450} was measured.

Comparison of adhesive abilities was performed by using Student's unpaired *t*-test. A P value <0.05 was considered as statistically significant.

Collagen Gel Contraction

Experiments were performed essentially as described previously.[17] Briefly, 24-well tissue culture plates were pre-coated with BSA. Trypsinized fibroblasts were suspended in MCDB medium (Sigma) and mixed with collagen solution [one part 0.2 mol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.0; four parts collagen (Vitrogen-100; 3 μg/ml); and five parts MCDB $\times$2] yielding a final concentration of 80,000 cells/ml and 1.2 mg/ml collagen. Collagen/cell suspension (1 ml) was added to each well. After polymerization, gels were detached from wells by adding 1 ml of MCDB medium. Contraction of the gel was quantified by loss of gel weight and decrease in gel diameter over a 24-hour period. Comparison of collagen gel contraction was performed by using Student's unpaired *t*-test. A P value <0.05 was considered statistically significant.

Proteoglycan Biosynthesis Inhibition

For experiments of proteoglycan biosynthesis inhibition, cells were cultured for 4 days in DMEM, 10% FBS with 1 mmol/L α-D-xyloside (4-methylumbelliferyl-α-D-xyloside; Sigma) as a control or 1 mmol/L β-D-xyloside (4-methylumbelliferyl-β-D-xyloside; Sigma), which blocks heparin sulfate side chain formation and proteoglycan biosynthesis.[20–22] The cells were then washed twice in PBS before further experimentation. Inhibitor toxicity was assessed using a cell viability assay as described by the manufacturer (MTT; Roche, Laval, Quebec).

Cell Transfections

Fibroblasts (NIH 3T3; ATCC, Manassas, VA) were transfected using Polyfect (Qiagen, Crawley, United Kingdom) essentially as previously described.[6,13,16] Briefly, fibroblasts were seeded into 6-well plates and transfected at ~50% confluence with a generic MEK/ERK-responsive promoter (Clontech, Palo Alto, CA; 0.5 μg/well) with either empty expression vector or expression vector encoding syndecan 4 (15; 1 μg/well). Cells were cotransfected with a CMV promoter-driven β-galactosidase reporter gene (Clontech; 0.25 mg/well) expression from which (Applied Biosystems, Foster City, CA) was used to adjust for differences in transfection efficiencies among wells. After transfection, cells were maintained in DMEM, 0.5% calf serum (Life Technologies) for 48 hours. Reporter gene expression vas determined (Applied Biosystems), normalized for differences in transfection efficiencies, and expressed as average $\pm$ SD (Student's paired *t*-test, $P < 0.05$). Experiments were performed twice, with six replicates.

siRNA Knockdown

Specific siRNA recognizing syndecan 4 was purchased through a kit containing a pool of several siRNA (Syndecan 4 SMARTPool; Dharmacon, Denver, CO). A recommended control siRNA labeled with a green fluorescent protein (GFP) tag (cyclophilin B; Dharmacon) was also purchased. Normal and dSSc fibroblasts were transfected using an electroporator and an optimized kit for primary fibroblasts (Nucleofector; Amaxa, Cologne, Germany). Cells were transfected either with control siRNA or control siRNA with syndecan 4 siRNA. Cells were fixed in 4% paraformaldehyde (15 minutes; Sigma) and stained with mouse anti-phospho-ERK (Cell Signaling) and Texas Red anti-mouse (Jackson, West Grove, PA) antibody. Transfected cells were detected by looking for green cells (from the GFP tag of the control siRNA).

Results

Lesional dSSc Fibroblasts Show an Enhanced Ability to Adhere to and Contract ECM Relative to Nonlesional dSSc Fibroblasts and Fibroblasts from Normal Individuals

Scar tissue is characterized by the excessive production of collagen. To begin to investigate the basis of the contribution of the fibroblast in dSSc to scar formation in dSSc, we used Western blot analysis with an anti-type I collagen antibody to compare the abilities of dermal fibroblasts from healthy individuals and dermal fibroblasts from unaffected and affected areas of dSSc patients to produce type I collagen. To our surprise, we found that although dSSc fibroblasts produced elevated levels of type I collagen, nonlesional (clinically unaffected) dSSc fibroblasts produced a level of type I collagen intermediate between lesional (clinically affected) dSSc fibroblasts and normal fibroblasts (Figure 1). Thus, we concluded that substantial differences in type I collagen levels per se were not directly responsible for the outward appearance of scar tissue in dSSc patients.

To further address the basis of the contribution of the lesional dermal dSSc fibroblast to scar formation in dSSc, we directly compared the abilities of dermal fibroblasts from healthy individuals and dermal fibroblasts from unaffected and affected areas of dSSc patients to adhere to and contract ECM. To assess the abilities of normal, nonlesional and lesional dermal fibroblasts to adhere to ECM, we coated 96-well plates with fibronectin. Fibroblasts were cultured from three normal individuals and nonlesional and lesional areas of three individuals with diffuse SSc. Cells were detached from tissue culture dishes with ethylenediamine tetraacetic acid and allowed to adhere to fibronectin for 45 minutes. We found that fibroblasts cultured from lesional areas of dSSc patients showed markedly elevated adhesive ability, relative to fibroblasts cultured from normal donors and nonlesional areas of dSSc patients (Figure 2A).

We reasoned that because lesional dermal dSSc fibroblasts excessively adhered to ECM, they might be expected to excessively contract ECM, because both of these activities require the formation of focal adhesions between the cytoskeleton and ECM.[23,24] To compare the

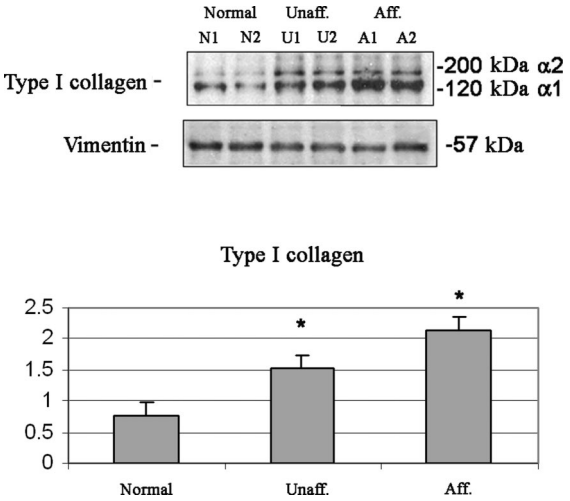


Figure 1. Both lesional and nonlesional dermal dSSc fibroblasts overexpress type I collagen. Equal amounts of conditioned media from normal (Normal) dermal fibroblasts (N1 and N2), nonlesional dSSc fibroblasts (Unaff., U1, and U2), and lesional dSSc fibroblasts (Aff., A1, and A2) were concentrated and subjected to Western blot analysis with an anti-type I collagen antibody, as described in Materials and Methods. Nonlesional dSSc fibroblasts (isolated from clinically unaffected areas of dSSc patients) display an intermediate level of type I collagen, compared with fibroblasts from normal, healthy individuals and lesional dSSc fibroblasts (isolated from clinically affected areas of dSSc patients). Six fibroblast lines from each cohort were used; a representative blot showing results from two individuals per cohort is shown. The bar graph indicates relative expression values of both chains of type I collagen, relative to vimentin [average ± SD ($N = 6$ individuals of each cohort); *, statistically significant difference from collagen expression in normal individual]. Both the two type I collagen chains (α and β) were detected using the antibody.

abilities of normal, nonlesional, and lesional dermal fibroblasts to contract ECM, we seeded dermal fibroblasts within a collagen matrix and allowed the resultant mixture to polymerize on a tissue culture plate. The solidified gel was then detached from the tissue culture plate and incubated for 24 hours in the presence of 0.5% FBS. After this period, we assessed collagen gel contraction by measuring the size and weight of the contracted gel.[17,25] We found that lesional dSSc dermal fibroblasts showed a greatly increased ability, relative to nonlesional dSSc and normal dermal fibroblasts, to contract a collagen matrix (Figure 2B). Collectively, these results suggest that the phenotypic features of the lesional dSSc fibroblast which intimately contribute to appreciable scar formation (ie, to the physical appearance of fibrotic lesions in dSSc skin) is not an elevation of collagen synthesis *per se*, but rather is at least partly due to an enhanced ability to adhere to and contract ECM relative to nonlesional and normal fibroblasts.

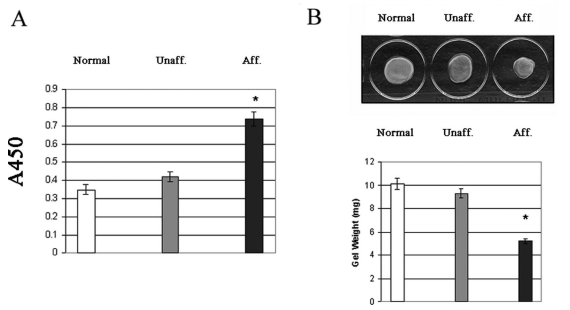


Figure 2. Lesional dSSc fibroblasts show enhanced adhesion and contraction of extracellular matrix. **A:** Normal (Normal), nonlesional (Unaff.), and lesional (Aff.) dSSc fibroblasts were allowed to adhere to BSA or fibronectin for 45 minutes, as described in Materials and Methods. Unbound cells were removed by washing. The remaining adherent cells were detected as described in Materials and Methods. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed in triplicate. Values shown are average ± SEM. *, significantly different from control ($P < 0.05$). **B:** Normal (Normal), nonlesional (Unaff.), and lesional (Aff.) dSSc fibroblasts were seeded into a floating collagen gel matrix and incubated for 24 hours as described in Materials and Methods. Weight of the contracted collagen gel was then measured. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed in triplicate. Values shown are average ± SD. *, significantly different from control ($P < 0.05$).

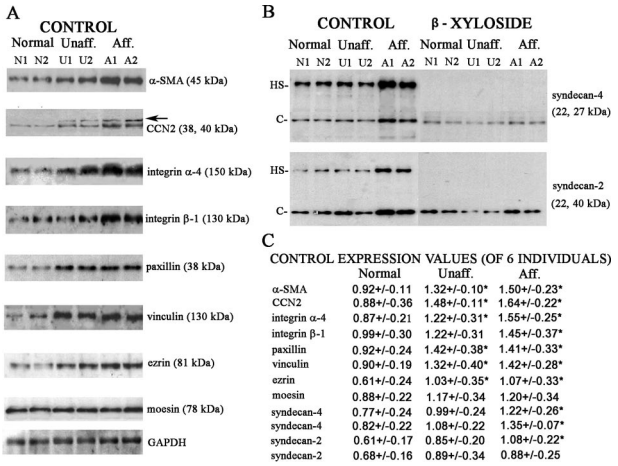
Lesional dSSc Fibroblasts Transcribe Elevated Levels of Proadhesive and Procontractile Genes

Based on our data showing that lesional dSSc fibroblasts showed a stable myofibroblast phenotype and displayed an enhanced ability to adhere to and contract ECM, we decided to explore further the basis for myofibroblast formation in fibrotic disease by investigating whether lesional dermal dSSc fibroblasts show elevated levels of expression of proadhesive and procontractile genes relative to normal dermal fibroblasts. Thus, we performed genome-wide transcriptional profiling of normal and lesional dSSc fibroblasts. We cultured fibroblasts obtained from biopsies of lesional areas of four scleroderma patients and fibroblasts taken from site-matched biopsies of three age- and sex-matched normal individuals. After reaching confluence, fibroblasts were serum-starved for 24 hours to avoid confounding influences of growth factors present in serum. RNAs were harvested and reverse transcribed into cDNA from which labeled cRNAs were generated to which were hybridized Affymetrix U133A arrays. Experiments were performed twice, excluded genes that were called absent or showed low expression values, and identified transcripts whose expression was up-regulated (more than threefold, $P < 0.01$) in lesional dSSc fibroblasts. (Approximately 350 up-regulated transcripts and 130 down-regulated transcripts were altered; not shown.) Given that we were interested in testing our hypothesis that lesional dSSc fibroblasts might overexpress proadhesive and procontractile genes, we focused our attention on genes known to have these properties. Transcripts involved with cell adhesion and contraction previously shown to be overexpressed in dSSc identified in our current study included those encoding CCN2 (connective tissue growth factor; Table 1),[13,26,27] which promotes cell adhesion to fibronectin.[19] That we were able to show that this transcript was elevated in our system served to validate our approach to identifying transcripts overexpressed in lesional SSc fibroblasts and gave credence to our subsequent analyses. SSc fibroblasts showed elevated expression of additional transcripts encoding proteins involved with cell adhesion, cell/matrix attachment such as several integrins and proteoglycans (Table 1). SSc fibroblasts expressed the proadhesive protein CCN1 (Cyr61), a protein structurally related to CCN2.[27] Lesional dSSc fibroblasts showed elevation of integrins α5, αV, and β3, which promote cell adhesion to

fibronectin and vitronectin. In addition, SSc fibroblasts showed elevated expression of the proteoglycans syndecans 2 and 4.[28–30] Finally, lesional dSSc fibroblasts overexpressed messages encoding procontractile proteins such as α-SMA, ezrin, and paxillin ([31–33]; Table 1). Thus, lesional dSSc fibroblasts showed elevated expression of mRNAs encoding proteins that would be expected to promote cell adhesion to and contraction of ECM, and thus we reasoned that overexpression of proadhesive and procontractile proteins by SSc fibroblasts might contribute to the fibrotic phenotype observed in this disorder.

Lesional dSSc Fibroblasts Overexpress the HSPGs Syndecan 2 and Syndecan 4

To further investigate the molecular mechanism underlying the progression to chronic fibrosis, we reasoned that genes found to be overexpressed in lesional dSSc fibroblasts, but not in nonlesional dSSc fibroblasts, relative to normal dermal fibroblasts might suggest a molecular basis for myofibroblast phenotype displayed by lesional dSSc fibroblasts. Thus, we performed Western blot analysis of cell lysates prepared from normal, nonlesional, and lesional dermal fibroblasts using antibodies against a cohort of proadhesive and procontractile proteins, including several predicted by our gene chip analyses to be overexpressed in dSSc lesional fibroblasts. We found that, in general, lesional and nonlesional dermal dSSc fibroblasts overexpressed proadhesive and procontractile proteins relative to normal dermal fibroblasts, with nonlesional fibroblasts displaying protein expression levels that were intermediate between normal and lesional dSSc fibroblasts (Figure 3, A and C). However, intriguingly, lesional dSSc fibroblasts displayed markedly elevated levels of expression of the HSPGs syndecan 2 and syndecan 4 relative to both nonlesional and normal fibroblasts (Figure 3, B and C). That syndecan 2 and syndecan 4 expression appeared to be markers of lesional dSSc fibroblasts suggested that the elevated expression levels of these HSPGs in lesional dSSc fibroblasts might contribute to the enhanced adhesive and contractile phenotype characteristic of lesional dSSc fibroblasts.



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CONTROL EXPRESSION VALUES (OF 6 INDIVIDUALS)

	Normal	Unaff.	Aff.
α-SMA	0.92+/-0.11	1.32+/-0.10*	1.50+/-0.23*
CCN2	0.88+/-0.36	1.48+/-0.11*	1.64+/-0.22*
integrin α-4	0.87+/-0.21	1.22+/-0.31*	1.55+/-0.25*
integrin β-1	0.99+/-0.30	1.22+/-0.31	1.45+/-0.37*
paxillin	0.92+/-0.24	1.42+/-0.38*	1.41+/-0.33*
vinculin	0.90+/-0.19	1.32+/-0.40*	1.42+/-0.28*
ezrin	0.61+/-0.24	1.03+/-0.35*	1.07+/-0.33*
moesin	0.88+/-0.22	1.17+/-0.34	1.20+/-0.34
syndecan-4	0.77+/-0.24	0.99+/-0.24	1.22+/-0.26*
syndecan-4	0.82+/-0.22	1.08+/-0.22	1.35+/-0.07*
syndecan-2	0.61+/-0.17	0.85+/-0.20	1.08+/-0.22*
syndecan-2	0.68+/-0.16	0.89+/-0.34	0.88+/-0.25

Figure 3. Western blot analysis of normal, nonlesional, and lesional SSc fibroblasts. **A:** Lesional and nonlesional dermal SSc fibroblasts overexpress proadhesive and procontractile proteins. Cell lysates (25 μg) from fibroblasts from two normal (Normal N1 and N2) individuals and nonlesional (Unaff. U1 and U2) and lesional (Aff. A1 and A2) areas of two individuals with diffuse SSc fibroblasts were subjected to Western blot analysis with anti-moesin, anti-vinculin, anti-CCN2, anti-ezrin, anti-α-SMA, anti-α4 integrin, anti-β1 integrin, and anti-GAPDH antibodies. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed. A representative blot showing results from two individuals of each cohort is shown. Please note that CCN2 is glycosylated and displays a characteristic slower migrating band at 40 kd (**arrow**) representing the glycosylated form of CCN2.[49] **B:** Lesional SSc fibroblasts overexpress sulfated syndecan 2 and syndecan 4. Cell lysates (25 μg) from dermal fibroblasts from two normal (Normal) individuals and nonlesional (Unaff.) and lesional (Aff.) areas of two individuals with diffuse SSc fibroblasts were subjected to Western blot analysis with anti-syndecan 2, anti-syndecan 4, and anti-GAPDH antibodies. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed. A representative blot is shown. A representative blot showing results from two individuals of each cohort is shown. Cells were treated with β-xyloside for 4 days to block heparin sulfate side chain formation and proteoglycan biosynthesis (β-xyloside) or α-xyloside, which permits side chain formation and proteoglycan biosynthesis as a control (Control). Note the faster migrating core proteins of syndecan 2 and 4 (C; 22 kd) and the slower migrating heparan sulfate-containing forms of syndecan 2 and 4 (HS; 40 and 27 kd, respectively). **C:** Quantitiative analysis of basal protein expression in normal, lesional SSc, and nonlesional SSc dermal fibroblasts. Cell lysates of fibroblasts from six normal individuals (Norm.) and six individuals with SSc [from nonlesional (Unaff.) and lesional (Aff.) areas of skin] were probed with antibodies outlined in **A** and **B**. Expression values, relative to GAPDH control, were obtained by densitometry. Average protein expression is shown (±SD; *, statistically different from control; $P < 0.05$).

Heparan Sulfate Proteoglycans Are Necessary for the Adhesive and Contractile Phenotype of Normal and Lesional dSSc Fibroblasts

HSPGs and proteoglycans possess heparin sulfate (HS) side chains, which interact with ECM components.[22,23] Thus, HS and HSPGs might be expected to promote ECM adhesion and contraction. To evaluate the hypothesis that the elevated levels of HSPG expression in lesional dSSc fibroblasts might contribute to the enhanced adhesive and contractile phenotype characteristic of lesional dSSc fibroblasts, we assessed whether blocking synthesis of HS side chains and HSPG synthesis might suppress the abilities of lesional dSSc fibroblasts to adhere to and contract ECM. Consequently, we cultured cells for 4 days in the presence of β-xyloside, which prevents the nucleation of HS side chain synthesis on HSPGs and proteoglycan synthesis (Figure 3B), or α-xyloside, a related analog that does not appreciably impact HS side chain nucleation or proteoglycan synthesis.[20–22] Expression of core protein (Figure 3B, bands indicated with letter C) was also reduced, consistent with the notion that β-xyloside reduces proteoglycan biosynthesis.[22] Adhesion and contraction assays were repeated as described above. We found that pretreatment of cells with β-xyloside potently reduced HS side chain formation and HSPG synthesis as visualized by Western blot analysis detecting syndecan 2 and 4 (Figure 3B). Pretreatment of cells with β-xyloside also reduced the enhanced ability of lesional dSSc fibroblasts to adhere to (Figure 4A) and contract (Figure 4B) ECM. We used a standard cell viability assay to show that under the conditions of the assay, β-xyloside and α-xyloside were not toxic (not shown). Interestingly, β-xyloside also reduced basal adhesion and contraction by normal and nonlesional fibroblasts, suggesting that HSPGs are generally required for the ability

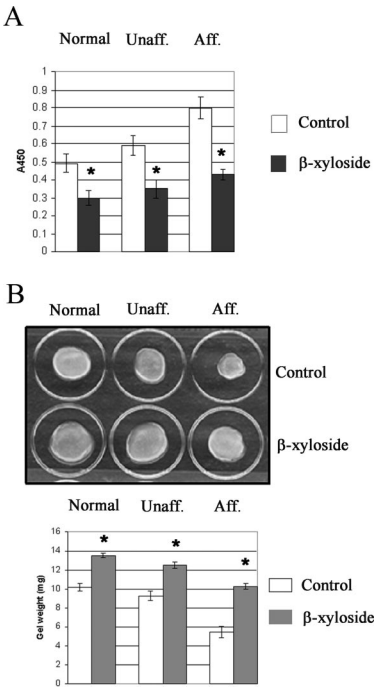

Figure 4. The elevated adhesive and contractile abilities of lesional SSc fibroblasts depends on heparin sulfate. **A:** Normal (Normal), nonlesional (Unaff.), and lesional (Aff.) dSSc fibroblasts were allowed to adhere to BSA or fibronectin for 45 minutes, as described in Materials and Methods. Unbound cells were removed by washing. The remaining adherent cells were detected as described in Materials and Methods. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed in triplicate. Values shown are average ± SEM. Cells were treated with β-xyloside for 4 days to block heparin sulfate side chain formation (β-xyloside) or α-xyloside, which permits side chain formation as a control (Control). Cells were treated with β-xyloside for 4 days to block heparin sulfate side chain formation (β-xyloside) or α-xyloside, which permits side chain formation as a control (Control). **B:** Normal (Normal), nonlesional (Unaff.), and lesional (Aff.) dSSc fibroblasts were seeded into a floating collagen gel matrix and incubated for 24 hours as described in Materials and Methods. Weight of the contracted collagen gel was then measured. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed in triplicate. Cells were treated with β-xyloside for 4 days to block heparin sulfate side chain formation (β-xyloside) or α-xyloside, which permits side chain formation as a control (Control). Values shown are average ± SD. *, significantly different from control ($P < 0.05$).

of fibroblasts to adhere to and contract ECM. Our results are consistent with the notion that the elevated expression levels of HSPGs greatly contribute to fibroblast adhesion and contraction, and to the enhanced adhesive and contractile abilities of lesional SSc fibroblasts, and that the enhanced lesional and contractile abilities of lesional dSSc fibroblasts are likely to arise through a dysregulation of pathways that mediate the abilities of normal (or nonlesional) fibroblasts to adhere to and contract matrix.

MEK/ERK Is Required for Maximal Contraction of ECM by Normal and Lesional dSSc Fibroblasts

We then sought to clarify the molecular mechanism through which HSPGs might promote contraction. Previously, we have shown that the ras/MEK/ERK cascade is important for cell spreading and the formation of actin stress fibers in fibroblasts.[19] Thus, we used Western blot analysis with an anti-phospho-ERK antibody to investigate whether the ras/MEK/ERK cascade was activated in SSc fibroblasts and whether β-xyloside treatment of SSc cells affected endogenous ras/MEK/ERK activation. We found that SSc fibroblasts displayed enhanced ERK phosphorylation that was dependent on HS, because preventing HS formation using β-xyloside markedly reduced the phosphorylation of ERK as visualized by Western blot (Figure 5A) or immunofluorescence (Figure 5B) analysis. Although nonlesional SSc fibroblasts displayed enhanced activation of ERK relative to normal fibroblasts, lesional SSc fibroblasts showed elevated endogenous ERK activation compared with nonlesional SSc fibroblasts (Figure 5). To assess the potential contribution of MEK/ERK to the excessive ECM contraction of lesional dSSc fibroblasts, we pretreated cells for 18 hours with U0126 before performing gel contraction assays. Because U0126 reduced ECM contraction by lesional dSSc fibroblasts, we concluded that the ras/MEK/ERK cascade was at least partially required for the ability of lesional fibroblasts to contract ECM (Figure 6). Similar results were obtained with the MEK inhibitor PD98059 (not shown). Interestingly, MEK blockade reduced basal adhesion and contraction by normal and nonlesional fibroblasts, suggesting that, in addition to HSPGs, the MEK/ERK MAP kinase cascade is also generally required for the ability of fibroblasts to adhere to and contract ECM. Our results are consistent with our previous impression derived from examining the effects of blocking HS formation on fibroblast ad-

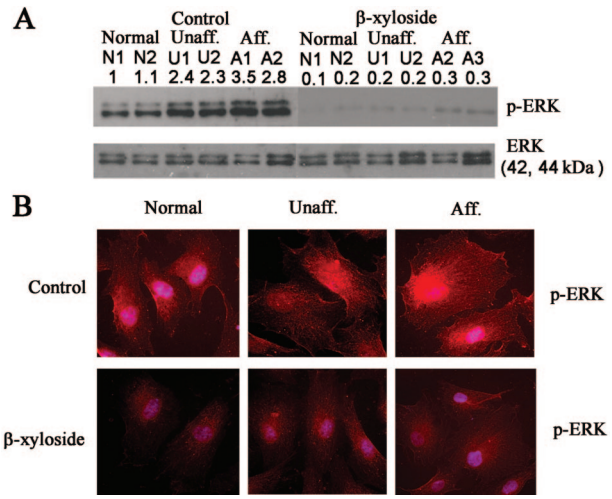

Figure 5. SSc dermal fibroblasts show elevated ERK activation, which depends on HS. **A:** Western analysis. Cell lysates (25 mg) from fibroblasts from two normal individuals (N1 and N2) and nonlesional (clinically unaffected, U1 and U2) and lesional areas (clinically affected, A1 and A2) of two individuals with diffuse SSc were subjected to Western blot analysis with anti-phospho ERK and anti-ERK antibodies. Cells were treated with β-xyloside for 4 days to block heparin sulfate side chain formation (β-xyloside) or α-xyloside, which permits side chain formation as a control (Control). Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed. A representative blot is shown. **B:** Immunofluorescence analysis. Cells treated as described in **A** were fixed in paraformaldehyde and stained with rabbit anti-phospho-ERK and Texas red-conjugated anti-rabbit antibodies as indicated. Cells were costained with 4′-6-diamidino-2-phenylindole (DAPI) (1 μg/ml) to detect nuclei (blue).

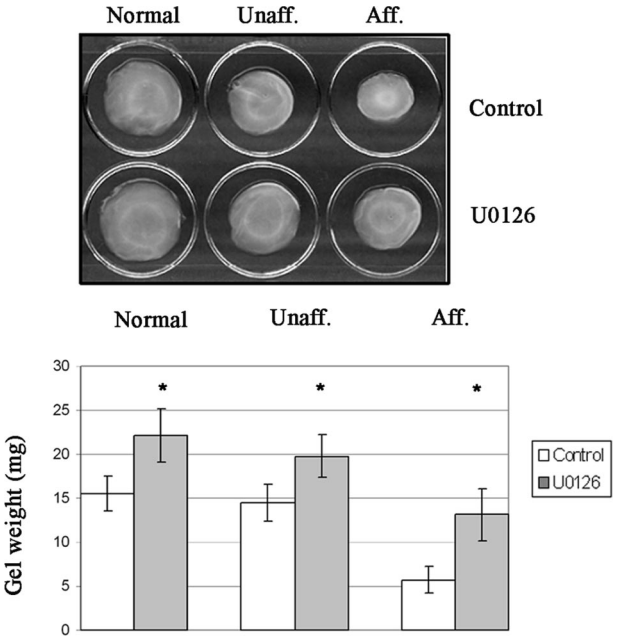

Figure 6. MEK activity is required for the enhanced contractile ability of lesional dSSc fibroblasts Normal, nonlesional, and lesional dSSc fibroblasts were seeded into a floating collagen gel matrix and incubated for 24 hours in the presence or absence of the MEK inhibitor U0126 (10 μmol/L) as described in Materials and Methods. Similar results were obtained using PD98059 (not shown). Weight of the contracted collagen gel was then measured. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed. Values are average ± SD. *, significantly different from control ($P < 0.05$).

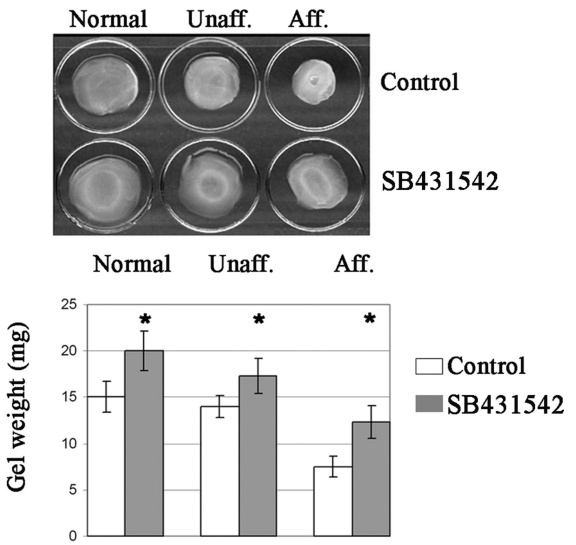

Figure 7. Lesional dSSc fibroblasts show enhanced ability to contract extracellular matrix that depends on ALK5. Normal (Normal), nonlesional (Non lesion.), and lesional (Lesion) dSSc fibroblasts were seeded into a floating collagen gel matrix and incubated for 24 hours as described in Materials and Methods in the presence or absence of the ALK5 receptor SB431542, as indicated. Weight of the contracted collagen gel was then measured. Dermal fibroblasts isolated from six normal individuals and lesional and nonlesional areas of six individuals with dSSc were assayed in triplicate. Similar results were obtained with SD-208 (not shown). Values shown are average ± SEM. *, significantly different from control ($P < 0.05$).

hesion and contraction that the phenotype of the lesional dSSc fibroblasts may result from an enhanced dysregulation of pathways that normally promote ECM contraction by healthy fibroblasts.

Antagonizing ALK5 Receptor Reduces the Adhesive and Contractile Ability of Normal and Lesional dSSc Fibroblasts

Previously, it has been shown that TGFβ is important for fibrotic responses in normal fibroblasts and in initiating fibrosis *in vivo* (for review, see reference 7). Furthermore, it has recently been shown that (TGFβ receptor type I) inhibition blocks the development of experimental lung fibrosis.[34] To address the potential contribution of endogenous TGFβ signaling, via the ALK5 receptor, to the fibrotic phenotype of dSSc fibroblasts, we assessed the abilities of an ALK5 receptor antagonist to alleviate the adhesive and contractile phenotype of normal, nonlesional, and lesional dSSc fibroblasts. We found that the enhanced adhesive and contractile ability of fibroblasts was markedly reduced by the ALK5 receptor antagonist (Figure 7, A and B). It is interesting to note that ALK5 inhibition also reduced the ability of normal fibroblasts to contract matrix, suggesting that endogenous signaling through ALK5 generally contributes to the ability of fibroblasts to contract ECM. Collectively, our results examining the effect of ALK5 inhibition on the adhesive and contractile abilities of fibroblasts are consistent with our previous observations that the phenotype of the lesional SSc fibroblast appears to represent an enhancement, or an exaggerated dysregulation, of the mechanism by which normal fibroblasts contract matrix. That is, the phenotypic features of the lesional dSSc fibroblast that intimately contribute to appreciable scar formation (ie, to the physical appearance of fibrotic lesions in dSSc skin)—namely the enhanced ability to adhere to and contract ECM relative to nonlesional and normal fibroblasts—depend on an exaggerated, more activated endogenous TGFβ signaling pathway acting through the ALK5 receptor and that this pathway also requires MEK/ERK and HSPGs.

Syndecan 4 Is Required for Fibroblast Contraction of ECM and Myofibroblast Formation

Our previous data suggested that TGFβ and HSPGs might cooperate, through MEK/ERK, to induce ECM contraction. This mechanism appeared to be operative in normal, lesional, and nonlesional dSSc fibroblasts, suggesting that similar procontractile pathways were operating in all three cell types, although there was likely to be a dysregulated, heightened response in lesional dSSc fibroblasts, resulting in enhanced ECM adhesion and contraction. We then wanted to uncover the common mechanism in fibroblasts by which TGFβ, HSPG, and ERK signaling was integrated. A clue as to the identity of a key, common integrator of these signals emerged from our earlier observations that the HSPGs syndecan 2 and syndecan 4 were overexpressed by lesional dSSc fibroblasts. Because our previous data suggested that the

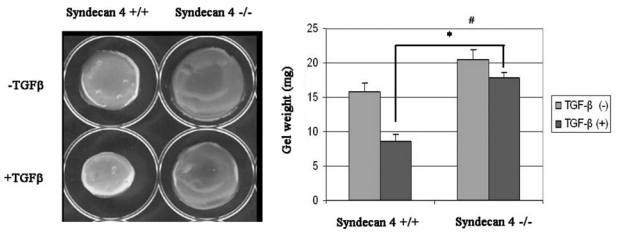

Figure 8. Collagen gel contraction by fibroblasts in response to TGFβ requires syndecan 4. Floating gel contraction assay. Syndecan $4^{+/+}$ and syndecan $4^{-/-}$ dermal mouse fibroblasts were seeded into a collagen gel matrix, and, after gel polymerization, the gel was detached from the tissue culture plate and incubated in the presence or absence of TGFβ1 (4 ng/ml) for 24 hours. Values shown are average ± SD. *, significantly reduced contractile response to TGFβ relative to control ($P < 0.05$). #, significantly reduced contractile response compared with syndecan $4^{+/+}$ fibroblasts.

enhanced contractile phenotype of lesional SSc fibroblasts might occur through an enhanced activation of pathways that promote contraction in normal cells, we hypothesized that syndecan 2 or syndecan 4 might be mediating the integration of TGFβ and ERK signaling in fibroblasts. A key aspect of adhesive and contractile signaling resulting in myofibrolast formation and wound closure is the formation of functional focal adhesions between the actin cytoskeleton and ECM. Because syndecan 4, but not syndecan 2, promotes focal adhesion formation[35] and *syndecan* $4^{-/-}$ mice show delayed wound closure,[36] we investigated whether syndecan 4 was capable of integrating procontractile signals provided from TGFβ. Thus, we believed it to be reasonable to assess whether syndecan 4 was generally required for ECM contraction by fibroblasts. To do this, we compared the ability of *syndecan* $4^{+/+}$ and *syndecan* $4^{-/-}$ mouse dermal fibroblasts to contract a collagen ECM with and without added TGFβ1. We found that loss of syndecan 4 significantly reduced the ability of cells to contract a collagen matrix both in the presence and absence of added TGFβ1 (Figure 8), suggesting that syndecan 4 is generally required by fibroblasts to contract ECM in response to TGFβ1. Similarly, we showed that *syndecan* $4^{-/-}$ cells displayed reduced ability to form α-SMA stress fibers, which are required to contract ECM, in response to TGFβ, as visualized by staining with an anti-α-SMA antibody (Figure 9).

Given that we were interested in ascertaining whether syndecan 4 was required for fibroblasts to integrate procontractile signals from TGFβ thorough ERK and given that previously we have shown that ERK activation is required for actin stress fiber formation[19] and matrix contraction (above), we first determined, by use of the MEK inhibitor U0126, that MEK/ERK activation was required for TGFβ-induced myofibroblast formation (Figure 9). We then determined that syndecan 4 was required for TGFβ-induced ERK activation, because this activity did not occur in syndecan $4^{-/-}$ dermal fibroblasts (Figure 10A). To assess whether overexpression of syndecan 4 by fibroblasts was sufficient to activate ERK in fibroblasts, we showed that transient transfection of an expression vector encoding syndecan 4 significantly activated a cotransfected generic MEK/ERK-responsive promoter/reporter construct compared with empty expression vector

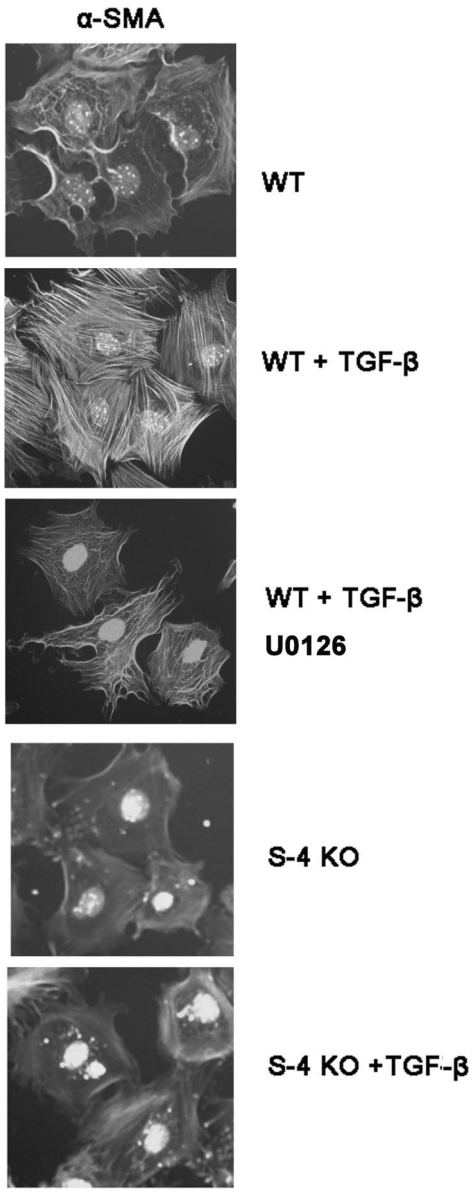

Figure 9. Syndecan 4 and ERK are required for myofibroblast induction by TGFβ. Syndecan $4^{+/+}$ (WT) and syndecan $4^{-/-}$ (S-4 KO) dermal fibroblasts were cultured, serum starved for 24 hours, and treated with TGFβ1 (4 ng/ml, 24 hours) in the presence or absence of a pretreatment with U0126 (10 μmol/L, 45 minutes). Similar results were obtained with PD98059. Cells were fixed in paraformaldehyde and stained with mouse anti-α-SMA antibody and a fluorescein isothiocyanate-conjugated anti-mouse antibody. Cells were counterstained with DAPI to detect nuclei.

(Figure 10B). Finally, siRNA recognizing syndecan 4 but not a control siRNA reduced the elevated ERK activation in dSSc fibroblasts (Figure 11). Our results suggest that syndecan 4 is essential for integrating procontractile signals from TGFβ by being required for the TGFβ induction of MEK/ERK. Collectively, these data are consistent with the notion that HSPGs are essential in fibroblasts for mediating profibrotic signals via TGFβ through ERK, and they suggest that antagonizing ALK5, MEK/ERK, HS formation, or syndecan 4 are likely to be of antifibrotic benefit.

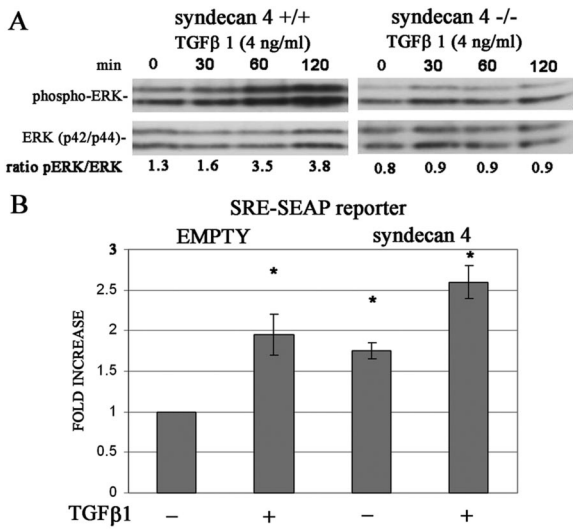

Figure 10. Syndecan 4 mediates the TGFβ1 induction of ERK. **A:** Syndecan 4 is necessary for the TGFβ1 induction of ERK. *Syndecan* $4^{+/+}$ and *syndecan* $4^{-/-}$ dermal fibroblasts were cultured, serum starved for 24 hours, and treated with TGFβ1 (4 ng/ml) for the durations indicated. Cell extracts were prepared, and equal amounts of lysate were subjected to Western blot analysis with anti-phospho-ERK and anti-ERK antibodies and horseradish peroxidase-conjugated secondary antibodies. The TGFβ induction of ERK phosphorylation does not occur in *syndecan* $4^{-/-}$ fibroblasts. **B:** Overexpression of syndecan 4 potentiates basal and TGFβ-induced ERK-dependent reporter activity. Cotransfection of expression vector encoding syndecan 4 (syndecan 4) activates a generic MEK/ERK-responsive promoter (serum response element-secreted enhanced alkaline phosphate (SRE-SEAP)) in fibroblasts, compared with empty expression vector (EMPTY). After a serum starvation step, cells were incubated in the presence or absence of TGFβ for an additional 24 hours. Average ± SD of representative experiment is shown ($N = 6$; *, significantly different from control; $P < 0.05$).

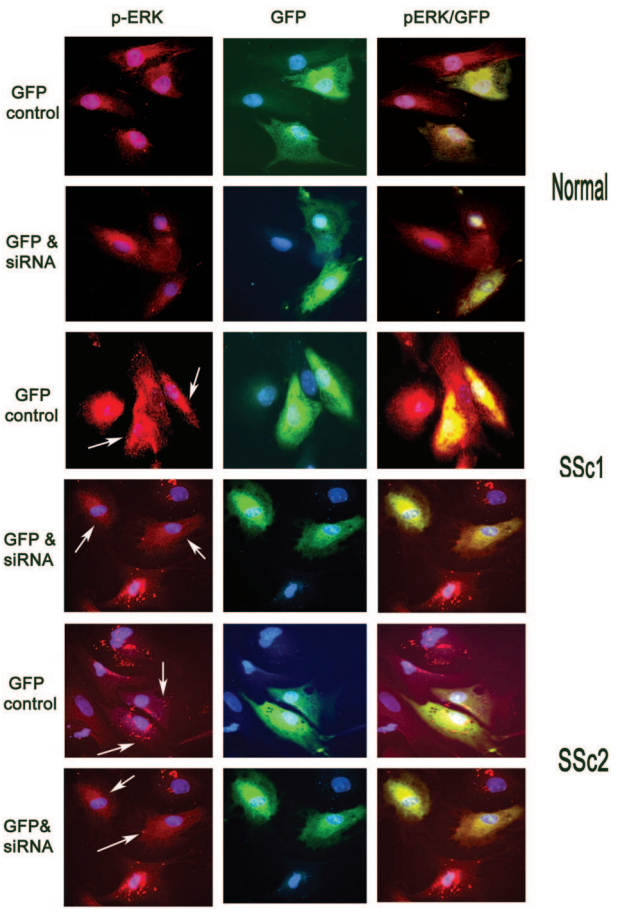

Figure 11. Syndecan 4 mediates the elevated activation of ERK in SSc fibroblasts. Knockdown of syndecan 4 reduces ERK activation in SSc fibroblasts. Normal and SSc fibroblasts (from two independent patients: SSc 1 and SSc 2) were transfected with either a GFP-tagged control siRNA or the GFP-tagged control siRNA (cyclophilin B) with siRNA recognizing syndecan 4. Cells were fixed, and phosphorylated ERK epitopes were detected by indirect immunofluorescence. Transfected cells were detected by their green color (due to the GFP-tagged control siRNA). SSc cells (SSc1 and SSc2) receiving syndecan 4 siRNA (GFP&siRNA) showed reduced ERK activation (**arrows**) relative to untransfected controls (no arrow) and cells transfected with GFP-tagged control siRNA (GFP control, **arrow**).

Discussion

Pathological chronic fibrosis is characterized by the elevated production and contraction of ECM. There is no effective treatment for pathological fibrosis. Although the effecter cell of chronic fibrosis is believed to be the fibroblast, the key molecular features of the fibroblast that result in appreciable, clinically defined scar tissue are not known. Thus, which aspects of the fibroblasts present in fibrotic lesions to target in antifibrotic therapies is unclear. Because the systemic disease dSSc affects many internal organs and the skin, studying the molecular basis of scleroderma is likely to yield insights into the basis of fibrotic disease in general. Isolation of fibroblasts from dSSc and normal skin is straightforward, and thus the contribution of the dSSc fibroblast to the phenotype of fibrosis can be readily ascertained. There are several novel results described in our current report. First, our results suggest that the phenotypic difference between fibroblasts from nonlesional and lesional skin that results in appreciable, clinically defined scarring is not the elevated production of collagen matrix per se, but rather an enhanced ability of the fibroblast from scarred areas to adhere to and contract ECM. Second, our results show that HS side chains (of HSPGs) are required for the ability of fibroblasts to adhere to and contract ECM. Third, antagonism of HS side chain formation alleviates the enhanced ability of lesional dSSc fibroblasts to adhere to and contract ECM. Fourth, the enhanced contractile ability of dSSc fibroblasts depends on MEK/ERK and ALK5/TGFβ. Interestingly, ERK, ALK5, or HSPG side chain antagonism also seemed to suppress the ability of normal fibroblasts to contract matrix, suggesting that the phenotype of the lesional SSc fibroblast results from a hyperactivation of pathways that control the ability of normal, healthy fibroblasts to contract ECM. Although, in general, nonlesional dSSc fibroblasts show an elevated expression of profibrotic proteins, lesional dSSc show elevated expression of the HSPGs syndecan 2 and 4, proteins that are involved with cell adhesion and cytoskeletal assembly.[22–24] Given that lesional fibroblasts appeared to shown enhanced ECM contraction by a mechanism that represented an exaggerated dysregulation of the pathways that normally control the ability of fibroblasts to contract ECM, we went on to show that syndecan 4 was generally required for fibroblasts to integrate the procontractile signals from TGFβ by being required for the ability of TGFβ to activate ERK in fibroblasts and to induce fibroblasts to contract ECM. Collectively, these results

suggest that the presence of pathological, clinically defined scarring in chronic fibrotic disease is primarily due to enhanced ECM contraction by a dysregulation of a pathway mediating ECM contraction by normal fibroblasts and that antagonizing MEK/ERK, ALK5 inhibitors, syndecan 4, or HS side chain formation might be useful in combating chronic fibrotic disease.

Although scar tissue is characterized by an increase in ECM deposition and contraction by fibroblasts, the exact phenotypic alteration of fibroblasts that results in persistent scar tissue deposition is not known. Accordingly, we report here the first detailed, phenotypic characterization of lesional, nonlesional, and normal fibroblasts. Consistent with a previous study in which whole skin, but not individual cell types, was examined,[37] we found that alterations of the phenotype of the dSSc fibroblast occurred before the formation of clinically detectable scar tissue. In general, we found that nonlesional dSSc fibroblasts showed an elevated expression of profibrotic proteins including type I collagen, albeit at levels intermediate between normal and lesional dSSc dermal fibroblasts. Thus, elevated ECM deposition per se might not be sufficient to promote active, appreciable scar formation *in vivo*. However, our findings that lesional dSSc fibroblasts excessively adhered to and contracted ECM relative to both normal and nonlesional dSSc dermal fibroblasts suggests a functional basis for the appearance of appreciable clinically involved scar tissue. Our results further suggest that a feature of lesional dSSc fibroblasts appears to be stably differentiated into a profibrotic phenotype; however, immunofluorescent staining of cells with profibrotic markers suggest a phenotypic heterogeneity in the dSSc cell population, suggesting that a clonal expansion of a subset of profibrotic cells may result in the fibrotic phenotype of dSSc.

Although TGFβ principally signals through Smads, recent data has shown that ras/MEK/ERK MAP kinase cascade modifies TGFβ signaling.[7] For example, the TGFβ induction of CCN2 in mesangial cells and fibroblasts requires ras/MEK/ERK.[38–40] The synthetic prostacyclin Iloprost, which alleviates symptoms of fibrosis in scleroderma patients, acts at least in part by antagonizing the TGFβ induction of MEK/ERK.[40,41] However, the overexpression of CCN2 in SSc fibroblasts is independent of TGFB.[26,42] In addition to the effects on cell proliferation,[43] we have recently shown that MEK/ERK is required for fibroblast spreading and actin stress fiber formation in fibroblasts.[19] Although it has been previously established that TGFβ promotes ECM contraction,[42] the molecular mechanism underlying this process is largely unknown. In addition, has not been understood how TGFβ induces ERK activation. Our results suggest that TGFβ induction of myofibroblasts depends on syndecan 4 to activate ERK. These results are consistent with previous data that wound closure is delayed in *syndecan* $4^{-/-}$ mice,[36] and they extend previous data showing that *syndecan* $4^{-/-}$ fibroblasts display reduced FAK phosphorylation in response to adhesion to fibronectin.[15] Intriguingly, syndecan 4 is induced during wound repair of neonatal but not fetal skin, suggesting that syndecan 4 expression correlates with scarring potential.[44] That syndecan 4 is required for myofibroblast formation suggests that antagonizing action of syndecan 4, for example by using soluble syndecan 4, might be useful in developing antifibrotic therapies. Syndecan 4 might directly act as a coreceptor for TGFβ, or syndecan 4 might work indirectly by affecting expression of genes required for the ability of TGFβ to activate ERK. Our data do not distinguish between these possibilities, which are currently under investigation. It is interesting to note that the addition of xyloside, which blocks HS side chain addition, also reduces proteoglycan biosynthesis,[20–22] including a partial reduction in core protein expression (Figure 3B). HS side chains can mediate growth factor-dependent and adhesive signaling, both of which can influence downstream gene expression.[22,23,35,36,45] Thus the reduction in HS formation observed with β-xyloside treatment of fibroblasts might indirectly influence core proteoglycan expression, reflecting this fact. Whether xyloside treatment affects the expression of profibrotic genes in SSc cells is currently under investigation. That said, our results suggest that syndecan 4 is required in fibroblasts to integrate TGFβ/ERK signals to promote α-SMA stress fiber formation and ECM contraction and is consistent with the notion that that this pathway is dysregulated in fibrosis, leading to the enhanced ECM contraction characteristic of pathological scarring in SSc. It is interesting to note that whereas normal and nonlesional fibroblasts express similar amounts of syndecan 2 and 4, nonlesional fibroblasts show increased ERK activation relative to normal fibroblasts, albeit at lower levels than lesional SSc fibroblasts. These observations presumably reflect that the enhanced ERK activation in nonlesional SSc fibroblasts must be independent of enhanced syndecan expression but must be due to additional pathways, reflecting the fact that SSc and fibrotic disease are likely to be multifactorial.

The contribution of TGFβ and TGFβ signaling to fibrosis, including SSc, has been confusing. In fibrosis, there does not appear to be consistent elevation of TGFβ ligand within the scar; in SSc, elevated TGFβ levels appear to be associated with the portion of the scar at the border between the nonlesional and lesional area.[46] In addition, application of TGFβ ligand results in transient fibrotic responses *in vivo*.[47] This has led to the notion that mechanisms in addition to simply the TGFβ ligand are involved with the pathogenesis of SSc.[7,48–50] Recent observations have suggested that although secreted proteins such as endothelin-1[17] and CCN2,[14,48] in addition to TGFβ, appear to play key roles in pathological fibrosis, dysregulation at the level of cell surface receptors also appears to play key roles in fibrotic disease, perhaps by mediating sustained, enhanced, or dysregulated responses to TGFβ ligand. That is, alterations in TGFβ levels per se need not necessarily be required to have this ligand promote sustained fibrosis *in vivo*. Instead, alterations in the ratio between TGFβ RI and TGFβ RII may contribute to sustained fibrosis[8] or in enhanced expression of HSPGs/syndecan 4 (this report). Interestingly, such dysregulation of TGFβ signaling pathways may occur even the absence of a role of TGFβ ligand.[49] In any event, our results suggest that enhanced dysregu-

lation of TGFβ signaling pathways through ALK5 seems to play a key role in the pathogenesis of chronic fibrosis in dSSc.

In conclusion, we have shown that the lesional SSc fibroblast possesses an autonomous phenotype and that the lesional SSc fibroblast intimately contributes to the excessive scarring in SSc by excessively contracting and adhering to ECM. We found that maximal ECM contraction by fibroblasts requires HSPGs, TGFβ, and ERK and that this pathway is dysregulated in SSc fibroblasts. Our results suggest that targeting syndecan 4, ALK5, or ras/MEK/ERK may yield new methods of preventing scar tissue formation in chronic fibrotic disease.

Acknowledgments

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